A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules

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In B cells, the non-classical human leukocyte antigens HLA-DO (DO) and HLA-DM (DM) are residents of lysosome-like organelles where they form tight complexes. DM catalyzes the removal of invariant chain-derived CLIP peptides from classical major histocompatibility complex (MHC) class II molecules, chaperones them until peptides are available for loading, and functions as a peptide editor. Here we show that DO preferentially promotes loading of MHC class II molecules that are dependent on the chaperone activity of DM, and influences editing in a positive way for some peptides and negatively for others. In acidic compartments, DO is engaged in DR–DM–DO complexes whose physiological relevance is indicated by the observation that at lysosomal pH DM–DO stabilizes empty class II molecules more efficiently than DM alone. Moreover, expression of DO in a melanoma cell line favors loading of high-stability peptides. Thus, DO appears to act as a co-chaperone of DM, thereby controlling the quality of antigenic peptides to be presented on the cell surface.

Keywords: antigen presentation/chaperone/editing/HLA-DR/peptide binding

Introduction

Classical class II molecules are polymorphic receptors specialized in binding a highly diverse set of self and foreign peptides for recognition by CD4⁺ T cells. In order to meet antigens taken up via the endocytic pathway, conventional class II heterodimers are sorted from the endoplasmic reticulum (ER) to endosomal/lysosomal organelles with the help of the invariant chain (Ii) (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990). Ii chaperones the peptide-binding groove of $\alpha\beta$ dimers by virtue of a segment called CLIP (class II-associated Ii peptide) (Riberdy *et al.*, 1992; Bijlmakers *et al.*, 1994) and, in addition, uses N- and C-terminal flanking regions of CLIP for the formation of $\alpha\beta$ Ii complexes (Vogt *et al.*, 1995).

Following proteolysis of Ii in early endocytic compartments (Maric *et al.*, 1994; Amigorena *et al.*, 1995), CLIP remains complexed with $\alpha\beta$ dimers (Avva and Cresswell, 1994) and must be released before loading with antigenic peptides can occur (Roche and Cresswell, 1990). CLIP dissociation is favored at low pH due to a self-release mechanism exerted by its N-terminal tail (Urban *et al.*, 1994; Kropshofer *et al.*, 1995a,b) but, owing to the polymorphic nature of the ligand-binding site of conventional class II molecules, there are strong allelic differences in the affinity and the off-rate of CLIP (Liang *et al.*, 1995; Sette *et al.*, 1995).

The need to overcome class II allelic differences in the efficiency of self-release of CLIP may be one reason why the non-classical major histocompatibility complex (MHC) class II molecule HLA-DM (DM) evolved (Fling *et al.*, 1994; Morris *et al.*, 1994). In contrast to classical MHC II dimers, DM and its murine counterpart, H-2M, do not present peptides on the cell surface. A sorting signal in the cytosolic tail directs DM and H-2M to endosomal/lysosomal compartments without the involvement of Ii (Lindstedt *et al.*, 1995; Marks *et al.*, 1995). In lysosome-like vesicles, such as so-called MHC class II compartments (MIICs; Peters *et al.*, 1991), DM was found to co-localize and co-precipitate with classical $\alpha\beta$ dimers (Sanderson *et al.*, 1994, 1996; Kropshofer *et al.*, 1997b). However, in murine A20 B cells, H-2M was also described in lysosomal organelles devoid of classical MHC II molecules (Pierre *et al.*, 1996). A transient interaction between DM and DR molecules appears to be responsible for the removal of CLIP and the enhancement of peptide binding (Denzin and Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995), thereby attaining turnover numbers of up to 12 DR–CLIP complexes per minute at endosomal pH (Vogt *et al.*, 1996). Consequently, the lack of DM in cells leads to an accumulation of $\alpha\beta$ –CLIP complexes (Riberdy *et al.*, 1992; Sette *et al.*, 1992; Miyazaki *et al.*, 1996) and to strongly impaired presentation of protein antigens (Mellins *et al.*, 1990; Fung-Leung *et al.*, 1996; Martin *et al.*, 1996; Miyazaki *et al.*, 1996). DM also removes non-CLIP peptides, provided that they display an intrinsically low kinetic stability (Kropshofer *et al.*, 1996; Weber *et al.*, 1996): class II–peptide complexes undergo continued proofreading by DM, so that self or antigenic peptides with suboptimal anchor side chains or of inappropriate length are removed (Kropshofer *et al.*, 1996; van Ham *et al.*, 1996; Weber *et al.*, 1996). Thus, DM functions as a peptide editor that selects for high stability class II–peptide complexes (Sloan *et al.*, 1995; Kropshofer *et al.*, 1997a). Moreover, in lysosomal compartments of B cells, a considerable fraction of DM remains bound to empty DR dimers in order to prevent their aggregation or denaturation at low pH (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997b). Consistent with earlier reports, these data

suggest that there may be a shortage of appropriate peptides in loading compartments (Germain and Hendrix, 1991; Sadegh-Nasseri and Germain, 1991). Consequently, especially those empty class II allelic products that have a strong tendency to dissociate or aggregate benefit most strongly from being chaperoned by DM (Kropshofer *et al.*, 1997b). Consistent with the chaperone and editing activity described *in vitro*, H2-M was revealed to have an essential, li-independent function during antigen processing and presentation *in vivo* (Tourne *et al.*, 1997; Kenty *et al.*, 1998; Kovats *et al.*, 1998; Swier *et al.*, 1998).

Two other non-classical class II genes, HLA-DNA and HLA-DOB, have been described (Tonelle *et al.*, 1985; Trowsdale and Kelly, 1985; Servenius *et al.*, 1987). In human B cells, the products of these genes, DN α and DO β , respectively, were found to form a heterodimer, designated HLA-DO (DO) (Liljedahl *et al.*, 1996; Douek and Altmann, 1997). DO forms tight complexes with DM in the ER and is thereby sorted to lysosomal vesicles (Liljedahl *et al.*, 1996; Douek and Altmann, 1997). The evolutionary conservation of HLA-DNA and HLA-DOB (Trowsdale, 1995), the tight association of DO with DM (Liljedahl *et al.*, 1996) and the selective expression of DO in antigen-presenting cells, such as B cells, dendritic cells and thymic epithelial cells (Douek and Altmann, 1997), point towards a role for DO in antigen presentation. In two studies, it was reported that DM–DO complexes fall short in catalyzing peptide loading at pH 5.0, suggesting that DO may act as a negative modulator of DM (Denzin *et al.*, 1997; van Ham *et al.*, 1997). However, from studies with B cells of mice lacking H2-O, the murine equivalent of DO, it was concluded that H2-O promoted presentation of antigens internalized by membrane immunoglobulin (Liljedahl *et al.*, 1998).

Here we show that DO can enhance the efficiency of peptide loading, especially in the context of class II alleles such as DR4 which depend on DM as a chaperone. DO was found to stabilize DM at low pH, thereby preserving its chaperone activity, and DO–DM complexes were superior to DM in protecting empty DR molecules. According to these lines of evidence, DO appears to serve as a co-chaperone of DM.

Results

DO is engaged in heterotypic DR–DM–DO complexes in MIICs

In lysosomal compartments, 20–25% of the DR molecules (Schafer *et al.*, 1996; Kropshofer *et al.*, 1997b) and most, if not all, DO molecules are associated with DM (Liljedahl *et al.*, 1996). However, it is not clear whether DO remains bound to DM when the latter is engaged in complexes with DR or whether DO prevents DM from interacting with DR, which might be one possible explanation of the negative regulation of DM activity by DO. To differentiate between these two alternatives, the human B-cell line WT-100 was subjected to Percoll density centrifugation, thereby separating subcellular organelles (Kropshofer *et al.*, 1997b). Dense fractions positive for lamp-1 and β -hexosaminidase, typical markers for lysosome-like organelles including MIICs, were homogenized in the mild detergent CHAPS. When we immunoprecipitated with the monoclonal antibody (mAb) DM.K8 and re-precipitated

with mAb L243, direct evidence for the physical association of DR, DM and DO was obtained (Figure 1A). In NP-40, only DR was precipitated, due to the dissociation of DM–DO from DR in this detergent, as described for DM (Sanderson *et al.*, 1996).

To determine the relative amount of DR–DM–DO complexes in lysosome-like loading compartments, CHAPS homogenates were subjected to serial immunoprecipitations. The anti-DR $\alpha\beta$ mAb L243 removed most of the DO and DM molecules (Figure 1B), but not another lysosomal resident protein, lamp-1 (data not shown). The anti-DO mAb DOB.L1 co-precipitated 60–70% of the DM, but only low amounts ($\leq 10\%$) of the DR molecules (Figure 1B). In agreement with the previously described 1:5 to 1:4 DM:DR ratio in MIICs (Schafer *et al.*, 1996; Kropshofer *et al.*, 1997b), the anti-DM mAb DM.K8 depleted 20–25% of DR and, importantly, all DO molecules (Figure 1B). Together, these results demonstrate that in MIICs of B cells the majority of DM is engaged in DR–DM–DO complexes.

DM–DO is superior to DM in mediating peptide loading depending on the DR allele

To explore the influence of DO on the activity of DM, we affinity-purified DM–DO complexes from human spleen solubilized in NP-40. The amount of DO associated with DM varied in individual preparations, such as P1–P4 (Figure 2, bottom), depending on the isolation procedure. The catalytic activity of P1–P4 was tested at pH 5.0 in a peptide loading assay involving fluorescently labeled HA(307–319) (here called HA) peptide and DR4– or DR1–CLIP complexes, or HSP65(3–13) peptide and DR3–CLIP (Kropshofer *et al.*, 1995a). There was poor loading of DR1 or DR4 with HA peptide in the presence of preparation P2 which contained no DO (Figure 2). Higher loading efficiency was obtained with P1 and P4 that contained intermediate amounts of DO, and maximal loading with P3 containing the highest amount of DO. Importantly, there were only small differences in loading of DR3 (Figure 2). When DM–DO complexes from lysosomal organelles of a B-cell line were utilized, there was also a clear correlation between DO content and loading capacity with DR4 (data not shown). These findings indicate that in the context of certain class II allelic products, DM–DO complexes can be superior to DM in facilitating loading with cognate peptide.

Recombinant DO enhances the loading capacity of DM but down-modulates the loading kinetics

To study mechanistic aspects of DO function, full-length recombinant DO (rDO) was generated in a baculovirus expression system. SDS–gel electrophoresis and Western blot analysis of affinity-purified rDO revealed one band corresponding to DO β and two bands corresponding to DO α , probably glycosylation variants (Figure 3A). The influence of rDO on peptide loading was evaluated by supplementing a DM isolate devoid of endogenous DO (preparation P2; Figure 2). A strong dose-dependent enhancement of loading was obtained (Figure 3B), whereas rDO alone had no effect. rDO itself did not bind any of several peptides tested in the absence or presence of DM (data not shown). Relatively high rDO doses had to be used, suggesting that not all of the rDO preparation was

Fig. 1. DO is engaged in complexes with DM and DR in lysosomal compartments of B cells. **(A)** DO, DM and DR form heterotypic complexes. The EBV-transformed B cell line WT-100 was subjected to subcellular fractionation on a 25% Percoll gradient (Kropshofer *et al.*, 1997b). Dense organelles (fractions 1–7, $\rho = 1.065$ – 1.085 g/ml) which were positive for the lysosomal markers lamp-1 and β -hexosaminidase were pooled and lysed in 1% (w/v) CHAPS. In the first step, the lysosomal homogenate was immunoprecipitated with the anti-DM mAb DM.K8 coupled to Sepharose beads. The beads were eluted at room temperature with 100 mM sodium phosphate, 50 mM sodium acetate, pH 5.0, supplemented either with 1% CHAPS (left panel) or 1% NP-40 (right panel). Eluates were adjusted to pH 6.8 by adding 1 M NaOH, and in a second step re-precipitated with the anti-DR α mAb L243. Each precipitate was probed for DO β , DM β and DR α by Western blot analysis with the mAbs DOB.L1, DM.K8 and the anti-DR α mAb 1B5, respectively. **(B)** DO is quantitatively associated with DM. Lysosomal organelles were lysed in 100 mM sodium phosphate pH 7.0, 1% CHAPS in the presence of protease inhibitors and depleted of DR, DO and DM by immunoprecipitation with the mAbs L243, DOB.L1 and DM.K8, respectively. As a negative control, the anti-A^k mAb H116-32 was used. After each of three consecutive rounds of depletion (30 min at room temperature), a representative aliquot of the supernatant was probed for residual DO β , DM β and DR α as described above. The precipitations were specific, as DOB.L1 did not deplete DO in the presence of the peptide used for generating the antibody, DM.K8 did not co-precipitate DR in the absence of DM and L243 did not deplete the lysosomal resident lamp-1 (data not shown). A typical titration with signals corresponding to 100–20 ng of DR is given (lower panel) in order to allow estimations of the efficiency of the depletions.

Table I. Catalytic parameters of DM-mediated peptide loading of DR4 in the absence and presence of rDO

DM	rDO	K_M^a (nM)	V_{max}^b (nM/min)	n^c (per min)
+	–	1210	210	8.5
+	+	230	110	4.2

^a K_M , Michaelis constant, is calculated from the intercept with the X-axis of the Lineweaver–Burk plot (Figure 3D). It denotes the concentration of DR4 at which half-maximal velocity of DM catalysis was reached.

^b V_{max} , maximal velocity, is calculated from the intercept with the Y-axis of the Lineweaver–Burk plot (Figure 3D). It denotes the velocity extrapolated for infinite concentrations of substrate.

^c n , turnover number, denotes the number of DR4 molecules that are loaded by one DM molecule/min. n is calculated by V_{max} divided by the concentration of DM.

biologically active. It is unlikely that the results were due to misfolded or denatured rDO molecules, because denaturation by boiling or acid treatment rendered rDO inactive (Figure 3B). These data demonstrate that the poor DM activity of the P2 preparation was not caused by the isolation procedure but by the lack of DO, as suggested above (Figure 2).

Variation of the pH revealed that rDO promoted loading of DR4 most efficiently at pH 4.5–5.5, which is the pH range typical for lysosomal or pre-lysosomal compartments, but not at higher pH values (Figure 3C). This could be confirmed with human DR1 and murine A^b from T2 cells (Figure 3C) as well as with DR1 from wild-type B cells (data not shown). These findings suggest that DO preferentially promotes peptide loading in acidic compartments of the MHC type which is compatible with

the fact that DO is a lysosomal resident protein (Liljedahl *et al.*, 1996).

The effect of rDO on loading was also assessed kinetically, as previously described for DM (Vogt *et al.*, 1996): we determined the initial velocity of HA-peptide binding to DR4 at various DR4 concentrations in the presence of DM with and without rDO. Double reciprocal plots according to Lineweaver–Burk resulted in linear regression curves (Fersht, 1985). From these curves, we could deduce that rDO reduced the Michaelis constant K_M from 1210 to 230 nM (Figure 3D; Table I), indicating that in the presence of rDO, a 5-fold lower concentration of DR4 was sufficient to obtain the same degree of loading as in the absence of rDO. This indicates that DR4 binds to DM–DO with higher affinity and/or stability than to DM. At the same time, rDO reduced the turnover number, a measure of the catalytic potential of DM, from $n = 8.5$ DR4 molecules/min to $n = 4.2$ (Figure 3D; Table I), which indicates that rDO down-modulates the DM-mediated catalysis of loading. Together, these data suggest that DM–DO complexes bind substrate molecules like DR4 more tightly than DM alone, which occurs at the expense of reduced turnover numbers.

Empty DR molecules are chaperoned more efficiently by DM–DO

Since DM has been shown to chaperone empty DR molecules (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997b), the enhancing effect of DO and its pronounced pH dependency might be due to improved stabilization of DR by DM–DO. DR4 molecules were pre-incubated for various times at pH 5.0 in the absence of peptide, and the fraction of surviving DR4 was then determined by its ability to still bind HA peptide. As shown in Figure 4A,

Fig. 2. Peptide loading in the presence of DM associated with a variable amount of DO. Total cell lysates from human spleen cells solubilized in 1% (w/v) NP-40 were passed over Sepharose beads coupled to the anti-DM mAb DM.K8. Depending on the stringency of the washing procedure (1 or 2% NP-40, 4 or 25°C, 20 or 60 min) prior to elution, different amounts of DO co-purified with DM, as shown for preparations P1–P4 by Western blot analysis with the mAbs DOB.L1 and DM.K8, respectively. Subsequently, the catalytic activity of DM preparations P1–P4 was tested by using physiologic DM:DR ratios: DR4, DR1 or DR3 (200 nM), purified from the respective T2 transfectants, and the respective DM preparations (20–40 nM) were incubated with AMCA-labeled peptide (10 μ M) at pH 5.0, 37°C for 15 min. AMCA-HA peptide was used for DR4 and DR1, AMCA-HSP65(3–13) peptide for DR3. The amount of peptide bound was determined by HPSEC (Kropshofer *et al.*, 1995a). The DM content of P1–P4 was determined by quantitative Western blot analysis using purified DM from T2 transfectants as a reference.

half of the DR4 molecules had lost their capacity to bind HA peptide after <5 min of pre-incubation of DR4–CLIP complexes at pH 5.0 in the absence of peptide. The presence of rDO alone during the pre-incubation period had no effect, and DM devoid of DO protected only modestly. However, DM associated with endogenous DO (Figure 2; fraction P3) prevented most of the inactivation. Thus, DM–DO complexes seem to chaperone DR4 more efficiently than DM alone. In retrospect, it is likely that the previously reported stabilization of DR by DM was performed with a mixture of DM and DM–DO because DO was co-purified inadvertently (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997b).

As DM is devoid of peptides (Kropshofer *et al.*, 1997b), DM itself may be inactivated at low endosomal/lysosomal pH like other empty class II molecules. Pre-incubation of DM without DO or DR at pH 5.0 confirmed this hypothesis: inactivation of DM proceeded with a half-time of ~90 min (Figure 4B). However, upon addition of rDO, almost 90% of the DM molecules remained catalytically active after 2 h. Control proteins, such as bovine serum albumin (BSA), DR3 or murine A^k, did not provide protection against inactivation (Figure 4C).

Impact of DO on peptide editing by DM

DM-induced dissociation of low-stability peptides is commonly referred to as ‘peptide editing’ and can be viewed as a manifestation of the chaperone function of DM. In the light of the improved chaperone activity of DM–DO complexes shown above, we investigated whether DO has an impact on DM acting as an editor. For this, we co-incubated DR4 with a mixture of eight synthetic peptides (Figure 5A) in the absence and presence of DM and rDO at pH 5.0. The DR4 molecules used were purified from T2 cells transfected with DR4 and DM (T2.DR4.DM), and the eight peptides were naturally processed self-peptides associated with DR4 (Rammensee *et al.*, 1995). Peptides that had been loaded onto DR4 were eluted by acid treatment and identified by means of their unique molecular weight, utilizing matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). In the absence of DM and DO, we found only small amounts of the eight exogenous peptides loaded onto DR4, and mostly the complex mixture of endogenous peptides from T2.DR4.DM, ranging from m/z = 1500–3000 (Figure 5B) was present. In the presence of DM, three peptides became dominant (Figure 5C): peptide 1 (from sphingolipid activator protein); peptide 5 (from cathepsin C); and, to a lesser extent, peptide 2 (from bovine transferrin). The positive effect of DM on binding of these three peptides was accompanied by release of endogenous peptides, as indicated by a reduction in area under the curve of the broad distribution of endogenous peptides (Figure 5C). This finding is characteristic for DM functioning as a peptide editor (Kropshofer *et al.*, 1996). Most importantly, in the presence of both DM and rDO, only peptide 5 remained dominant, followed by a new species, peptide 8 (from integrin VLA-4), whereas peptides 1 and 2 were strongly reduced (Figure 5D). In addition, the ‘mountain’ of endogenous peptides was diminished further. Similar results were obtained with DM–DO complexes purified from B cells (data not shown). These data demonstrate that fewer peptides resist DM-mediated editing in the presence of DO compared with DM alone, implying that DO renders the proofreading process more stringent. This finding is consistent with DO acting as a co-chaperone of DM.

Melanoma cells transfected with DO bear increased amounts of high-stability DR-peptide complexes

Given the positive impact of DO on loading of DR4 *in vitro* (Figure 2), we expected DR4 to benefit from DO *in vivo* as well. The human melanoma cell line M10 was stably transfected with DO α and DO β cDNAs. Compared with the parental M10 cells, M10.DO expressed strongly increased amounts of DO but similar levels of DR4 and DM (Figure 6A), reflecting a DO:DM ratio of ≤ 1 known from B cells (data not shown). Flow-cytometric analysis with the anti-CLIP mAb Cer.CLIP revealed that transfection with DO was accompanied by a partial reduction in surface presentation of CLIP (Figure 6B). Moreover, there were significant alterations in the repertoire of self-peptides imposed by DO, as shown by MALDI-MS (Figure 6C). The self-peptide profile from M10-derived DR4 molecules was rather broad and of high complexity, containing a subset of peptides with M_r = 2200–2700, some of which display typical masses of length variants of CLIP also recognized by the mAb Cer.CLIP (Figure 6B). This and

Fig. 3. rDO improves the activity of DM in a pH-dependent manner. (A) Affinity-purified rDO from a baculovirus expression system was run on an SDS gel (11.5%) and stained with Coomassie. For comparison, affinity-purified DR4 and DM from T2.DR4.DM transfectants are shown. In parallel, rDO was subjected to Western blot analysis and probed for DO β and DO α by utilizing the mAb DOB.L1 and an anti-DO α serum, respectively. The two DO α chains are likely to be glycosylation variants, as described (Liljedahl *et al.*, 1996). (B) DM (25 nM) from splenic preparation P2 (Figure 2) was co-incubated in the absence and presence of increasing amounts of rDO (100–400 nM) together with DR4 (100 nM) and AMCA-HA peptide (10 μ M) for 15 min at 37°C, pH 5.0. Peptide binding was monitored by HPSEC (legend to Figure 2). As a control, binding of HA peptide was determined without adding DM or rDO, in the presence of rDO eluted at pH 3.5 (a-rDO) or in the presence of rDO boiled for 2 min at 95°C (b-rDO). (C) The pH-dependence of DO-mediated enhancement was determined by incubating AMCA-HA peptide (10 μ M) with DR4 or DR1 (100 nM), or AMCA-E α (52–68) peptide (10 μ M) with A^b with and without DM (25 nM) and rDO (200 nM) at 37°C for 30 min. The indicated pH was adjusted by using 1 M acetic acid or 1 M NaOH. DM was purified from T2 transfectants. (D) The influence of DO on the kinetics of DM-mediated peptide loading was analyzed according to Michaelis and Menten in the following way: binding of AMCA-labeled HA peptide (20 μ M) to DR4 (60–500 nM) catalyzed by DM (25 nM) from T2 cells in the absence or presence of rDO (500 nM) was determined after 3 min of incubation at pH 5.0, 37°C by HPSEC, as described (Vogt *et al.*, 1996). Uncatalyzed binding did not exceed background levels (data not shown). Initial rates of loading were plotted versus the amount of DR4 in a double reciprocal diagram according to Lineweaver–Burk (Fersht, 1985). The catalytic parameters calculated from the linear regression curves are given in Table I. The correlation coefficient, $r^2 = 0.988$ (Δ) and $r^2 = 0.993$ ($\blacksquare$).

other subsets of peptides were lacking in the profile of M10.DO transfectants, where several other peptide species became dominant instead. This is reminiscent of the changes obtained with DO *in vitro* (Figure 5). Importantly, skewing of the peptide repertoire was also reflected by alterations in the capacity to exchange endogenous peptide for HA peptide, as almost 50% less HA peptide could be loaded onto DR4 purified from M10.DO compared with DR4 from M10 cells (Figure 6D). A probable explanation is that in M10.DO cells more endogenous peptides with high stability have been loaded which resist the exchange for exogenous HA peptide. Together, these data provide evidence that DO affects processing and presentation of peptides *in vivo*.

Discussion

There is evidence that DM is a chaperone of the endosomal/lysosomal system which stabilizes empty MHC II mole-

cules, thereby protecting them from unfolding and inactivation (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997b). After binding of appropriate peptide, class II molecules seem to acquire a conformational state that is incompatible with a stable interaction with DM. The earlier described potential to release CLIP and other low-stability peptides (Denzin and Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995; Kropshofer *et al.*, 1996) can be viewed as a consequence of the chaperone activity of DM (Kropshofer *et al.*, 1997b; Kovats *et al.*, 1998) and is consistent with the principles of kinetic proofreading (Kropshofer *et al.*, 1997a).

The non-classical class II molecule DO binds to DM in the ER and remains tightly bound even after having reached endocytic compartments (Liljedahl *et al.*, 1996) and so far, dissociation of DO from DM has not been reported. This finding raises the possibility that DO modulates the chaperone activity of DM. Our results suggest that DO functions as a co-chaperone of DM by

Fig. 4. DO prevents empty DR4 and DM from functional inactivation. DM-DO complexes are superior to DM in stabilizing empty DR4. DR4 (100 nM) from T2 transfectants was pre-incubated for 0–10 min at pH 5.0 at 37°C without addition of peptide in the absence or presence of rDO (500 nM) and/or DM (200 nM) from T2 transfectants or splenic DM-DO complexes (200 nM) from DM preparation P3 (Figure 2). After the indicated time of pre-incubation, the amount of functional DR4 molecules was quantified by adding AMCA-HA peptide (10 μ M) and by simultaneously supplementing the samples devoid of DM or DO with DM (200 nM; panels 1 and 2) or rDO (500 nM) (panels 1 and 3), respectively. Binding of HA peptide was determined by HPSEC after a 5 min incubation. One of two experiments with similar results is shown. DM P3 preparations from spleen and from T2 transfectants were normalized for equal DM contents by quantitative Western blot analysis. **(B)** DO prevents DM from functional inactivation. DM (25 nM) from T2 transfectants was pre-incubated at pH 5.0, 37°C in the absence and presence of 500 nM rDO for the indicated periods of time. Subsequently, the residual catalytic activity of DM was determined by adding DR4 (100 nM), AMCA-labeled HA peptide (10 μ M) and rDO (500 nM) in those cases where rDO was not present during the pre-incubation (left panel). Binding of AMCA-HA peptide was determined by HPSEC after 5 min incubation time. A maximum of 10–12% of the total amount of DR4 was occupied with AMCA-HA, as calculated according to a described procedure (Kropshofer *et al.*, 1995b). Representative results of two independent experiments are shown. **(C)** As a control, the experimental setup described above was used to pre-incubate DM (25 nM) for 1 h in the absence of other molecules or in the presence of rDO (500 nM), BSA (500 nM), A^k (500 nM) from T2 transfectants and DR3 (500 nM) from B-LCL COX. The data from two experiments are shown.

assisting DM in rescuing empty class II molecules. This view is supported by several findings: DM-DO complexes were superior to DM in preventing functional inactivation of DR4 (Figure 4A). This may be accomplished by DM-DO binding to DR4 more tightly than DM alone, as suggested by the observation that the K_M value of DR4 is considerably lower for DM-DO than for DM (Figure 3D; Table I). Moreover, for the class II alleles investigated, improved loading was only seen in the pH range 4.5–5.5 (Figure 3C), reflecting the pH of lysosomes or late endosomes where DM and DO accumulate (Liljedahl *et al.*, 1996) and where class II molecules tend to form aggregates (Germain and Rinker, 1993; Kropshofer *et al.*, 1997b). Finally, we could verify that in lysosomal subcellular fractions, including organelles of the MIIC type, the majority of DM indeed formed heterotypic complexes with DO and DR molecules (Figure 1). These complexes

Fig. 5. DO modulates DM-mediated editing. Mass spectrometric analysis of peptides bound to DR4 in the absence and presence of DM or rDO. **(A)** Purified DR4 (2 μ M) from T2.DR4.DM transfectants was incubated with a mixture of self-peptides (2 μ M each) **(B)** in the absence of DM and rDO, **(C)** in the presence of DM (0.4 μ M) from T2.DR4.DM, **(D)** or in the presence of DM (0.4 μ M) plus rDO (1 μ M) for 12 h at pH 5.0, 37°C. The peptide profile of DR4 prior to incubation with the self-peptide mixture is shown as a control **(B; inset)**. The input peptide mixture **(A)** contained eight synthetic peptides that were described as naturally processed DR4-associated self-peptides (Rammensee *et al.*, 1995): sphingolipid activator protein (165–176) (peptide 1; m/z = 1324), bovine transferrin (68–82) (peptide 2; m/z = 1694), HLA-A2 (103–117) (peptide 3; m/z = 1856), plasminogen activator inhibitor PAI-1 (261–281) (peptide 4; m/z = 1916), cathepsin C (170–185) (peptide 5; m/z = 1936), HLA-Bw62 (129–146) (peptide 6; m/z = 1963), AMCA-labeled PAI-1 (261–281) (peptide 7; m/z = 2131) and integrin VLA-4 (229–247) (peptide 8; m/z = 2351). Bound peptides were eluted by acid treatment and analyzed by MALDI-MS. Exogenous peptides are indicated by the respective numbers. Endogenous peptides from T2.DR4.DM give rise to the complex array of signals ranging from m/z = 1500–3000. For means of quantitation, an internal reference peptide, 500 fmol of synthetic Ii(183–193) from human Ii (sequence: EQKPTDAPPKE; indicated by an 'R' with m/z = 1238) was added to the samples prior to MALDI-MS analysis.

are supposed to contain empty DR molecules, which gain help from DM and DO as chaperone and co-chaperone, respectively, not excluding the possibility that DR molecules being loaded with low-stability peptide might still

Fig. 6. Transfection with DO promotes loading with high-stability peptides. The human melanoma cell line M10 was stably transfected with the genes coding for DO α and DO β and kept under selection without subcloning, thereby generating the polyclonal transfectant M10.DO. **(A)** M10 and M10.DO (1×10^6 cells) were lysed in 1% NP-40 and analyzed for DO, DM and DR by Western blot analysis. **(B)** Flow-cytometric analysis of M10 and M10.DO cells with mAb Cer.CLIP (anti-CLIP; black) and mAb ML11C11 (anti-DR $\alpha\beta$; gray). M10.DO cells display fewer CLIP–class II complexes on the cell surface than M10 cells. Staining was performed with goat anti-mouse IgG1-R-phycoerythrin. Background control was obtained with an irrelevant IgG1 mAb (white). M10.DO clones also displayed the CLIP-negative phenotype, whereas control transfectants of M10 were still positive for CLIP. **(C)** Mass spectrometry profiles of DR4-associated self-peptides from M10 and M10.DO cells. Peptides were eluted from purified DR4 molecules and analyzed by MALDI-MS, as described (Kropshofer *et al.*, 1997b). Compared with the M10 profile, the M10.DO profile contains several prominent peptide species at the expense of peptides with $M_r = 2300$ –2700, thereby pointing to altered peptide editing. **(D)** DR4 (100 nM) affinity-purified from M10 or M10.DO was co-incubated with AMCA-HA (50 μ M) with and without DM (10 nM) from T2 transfectants at pH 5.0 for 16 h and analyzed by HPSEC. Less AMCA-HA could be loaded onto DR4 purified from M10.DO compared with M10 cells. Values from two experiments are shown. The percentage occupancy was calculated from the amount of DR4, the co-eluting AMCA fluorescence and the specific fluorescence of AMCA-HA peptide, as described (Kropshofer *et al.*, 1995b).

engage in DR–DM–DO complexes. DO does not seem to be equally effective in the context of all class II alleles, as we observed DR4 and DR1 to benefit from DO, whereas only little effect was seen with DR3 in our *in vitro* loading assay (Figure 2). These allelic differences match the comparably high intrinsic resistance of DR3, moderate resistance of DR1 and very low resistance of DR4 to pH-induced inactivation, as reported previously (Kropshofer *et al.*, 1997b).

Another reason for the positive effects of DO may rest in the ability of DO to stabilize DM itself, as suggested by the observation that rDO prolonged the half-life of DM at pH 5.0 (Figure 4B). Conventional class II molecules are stabilized by peptide at later stages of their pathway, but since DM is unable to bind peptides (Kropshofer *et al.*, 1997b; unpublished data) it may be rendered more stable by its tight interaction with DO. When we compared different B-cell lines with regard to their DM and DO

levels, we found 50–90% of DM co-precipitating with DO (data not shown). The engagement with DO may assist DM survival in lysosome-like compartments, especially during the transit through those acidic compartments that are negative for conventional class II molecules (Pierre *et al.*, 1996). In turn, it is possible that the half-life of DO is prolonged through the association with DM, especially in light of the fact that DO appears to be unable to bind peptides either (unpublished data).

From previous work, it is obvious that DM alone is able to catalyze peptide loading (Sherman *et al.*, 1995; Sloan *et al.*, 1995; van Ham *et al.*, 1996). With regard to this catalytic activity, DO appears to down-modulate DM (Figure 3D): we found a reduced turnover number in loading of HA peptide onto DR4 in the presence of DO (Table I), indicating the possibility that in each catalytic round DM–DO remains bound to DR4 for a longer period of time compared with DM alone. This may be a direct

consequence of the tight interaction between DM–DO and the DR4 substrate, as implied by the 5-fold lower K_M (Figure 3D; Table I). Since, according to the kinetic proofreading model (Kropshofer *et al.*, 1997a), the stability of the DR–DM interaction is one parameter that determines which peptides will be released by DM, DO can be expected to modulate the editing function of DM as well. This hypothesis is substantiated by our finding that the DR4-associated self-peptide repertoire of the melanoma cell line M10 changed dramatically upon transfection with DO: several peptide species became predominantly presented at the same time the diversity of the self-peptide repertoire seemed to be reduced (Figure 6C). Moreover, the altered set of self-peptides selected under the influence of DO in M10.DO transfectants appeared to form more stable complexes with DR4, as the extent of exchange for exogenous HA peptide was lower compared with DR4 from parental M10 cells (Figure 6D). In an attempt to mimic the peptide editing process *in vitro*, we found that rDO further decreased the array of DR4-bound endogenous peptides compared with the profile obtained with DM alone (Figure 5C and D). At the same time, it favored binding of some but not all exogenously added peptides. Both findings demonstrate that DO modulates DM-mediated editing.

Importantly, rDO also facilitated the removal of two exogenously added peptides selected by DM in the absence of rDO (Figure 5C and D). This observation emphasizes that editing, by definition, can be either positive or negative for a given peptide (Katz *et al.*, 1996; Kropshofer *et al.*, 1997a), depending on the structural features of the respective peptide (Kropshofer *et al.*, 1996; van Ham *et al.*, 1996; Weber *et al.*, 1996). Negative editing of certain peptides by DO together with a reduction of the turnover number of DM by DO may explain why DO was described as an inhibitor of DM (Denzin *et al.*, 1997; van Ham *et al.*, 1997; Liljedahl *et al.*, 1998). In one of these studies, where overexpression of DO in Mel JuSo melanoma cells led to quantitative association of DM with DO, DO down-modulated DM-mediated peptide binding and still allowed significant levels of antigen presentation instead of blocking DM function (van Ham *et al.*, 1997). This observation was extended in another report, where association of DO with DM was suggested to limit the pH range in which DM is active to pH ≤ 5.0 , thereby promoting antigenic peptide loading in acidic compartments of the MHC type. In contrast to that, the third study reported that DM–DO complexes isolated from Raji cells were completely incapable of catalyzing peptide loading at pH 5.0 (Denzin *et al.*, 1997). However, when we isolated DM–DO from Raji cells, it was active and superior to DM in facilitating peptide loading of DR4 (data not shown). It has not yet been resolved whether the discrepant results are due to different isolation procedures or the type of peptides being used.

Expression of residual class II–CLIP complexes on the cell surface has been utilized as a cellular readout system for elucidation of the effect of DO on DM. Both Mel JuSo cells and the T-cell line CEM.CIITA (CEM transfected with CIITA) displayed strongly increased levels of class II–CLIP upon transfection with DO (Denzin *et al.*, 1997; van Ham *et al.*, 1997). However, in B cells of wild-type and H2-O-deficient mice, the surface expression of

A^b–CLIP was essentially identical (Liljedahl *et al.*, 1998). This is similar to the small reduction in DR4–CLIP levels upon transfection of M10 cells with physiological amounts of DO (Figure 6B). Moreover, in B-LCLs expressing similar levels of DR1 and equal levels of DM, there was an inverse correlation between the amount of DR1-associated CLIP and the total amount of DO (data not shown). Thus, apart from the probable relevance of allelic differences, it is possible that the CLIP^{high} phenotype of Mel JuSo.DO or CEM.CIITA.DO described above may have been facilitated by unphysiologically high amounts of transfected DO or unbalanced expression of DO α and DO β chains. We conclude from this that DO is unlikely to have a strong impact on DM-mediated removal of CLIP, but rather plays a role at later stages of the loading scenario.

According to our findings, DO is responsible for rendering DM as efficient as possible in chaperoning class II molecules. This may be relevant for antigen-presenting cells (APCs) expressing several allelic and isotypic class II molecules that may differ in their capacities to form complexes with DM, e.g. DR4 is loaded less efficiently than DR3 in the presence of DM alone. DO may aid in compensating these differences by acting as a co-chaperone. The co-chaperone function of DO appears to be especially crucial for peptide loading in lysosome-like compartments at low pH, where class II molecules tend to become functionally inactive. In agreement with this, H2-O-deficient mice displayed an impaired potential to process antigens internalized by membrane immunoglobulin into putative lysosomal compartments (Liljedahl *et al.*, 1998).

In summary, we have provided evidence that DO is able to promote DM-mediated peptide loading in an allele- and peptide-dependent way. Two mechanisms acting in a synergistic fashion may account for the effect of DO: (i) tight binding of DM to DO throughout the intracellular trafficking of both molecules preserves the activity of DM; and (ii) DO–DM complexes are superior to DM in chaperoning empty DR, suggesting that DO acts as a co-chaperone. This is reminiscent of the MHC class I presentation pathway where empty class I molecules are thought to be stabilized via class I–TAP–tapasin–calreticulin complexes in the ER (Solheim *et al.*, 1997). There is increasing evidence for the existence of chaperone–co-chaperone assemblies in biological systems; for example in the cytosol of eukaryotic cells, the heat shock proteins Hsp70 and Hsp90 work in concert with regulatory factors, designated Hip and Hop, in order to stabilize a multimeric chaperone complex that facilitates maturation of hormone receptors (Frydman and Höfheld, 1997). Likewise, it is conceivable that a specialized chaperone–co-chaperone complex such as DM–DO is necessary to ensure the survival of otherwise short-lived empty peptide receptors, namely class II molecules, prior to their encounter with antigen in acidic compartments where unfolding, denaturation and degradation are the predominant events.

Materials and methods

Cells

The Epstein–Barr virus (EBV)-transformed homozygous B-cell lines WT-100 (DR1Dw1) and COX (DR3Dw3), and the T $\times$ B hybrid cell line

T2 stably transfected with DR1Dw1 (T2.DR1), DR3Dw3 (T2.DR3), DR4Dw4 (T2.DR4), DR4Dw4 and DM (T2.DR4.DM) (Denzin *et al.*, 1994), murine A^k (T2.A^k) or A^b (T2.A^b), were maintained in roller bottles at 37°C in RPMI 1640 containing 20 mM HEPES and 10% heat-inactivated fetal calf serum (FCS) (Conco Lab. Division). *Spodoptera frugiperda* (SF9) cells (Pharmingen) were maintained in TNM-FH medium (Gibco-BRL) supplemented with 10% FCS at 27°C. For protein production, cells were grown in 500 ml spinner flasks in serum-free media BaculoGold (Pharmingen) or Ex-cell 400 (JHR Scientific) at 27°C.

Antibodies

The hybridoma cell lines L243 (anti-DR α β ; Lampson and Levy, 1980), 1B5 (anti-DR α ; Adams *et al.*, 1983), ML11C11 (anti-DR α β ; G.Moldenhauer, unpublished), DM.K8 (anti-DM β ; Vogt *et al.*, 1996) and Cer.CLIP [anti-CLIP(81–104); Denzin *et al.*, 1994] have been described. The rabbit DO α serum was prepared by immunizing rabbits with the C-terminal peptide from HLA-DO α (CMGTYSVSSVPR) coupled to keyhole limpet hemocyanin (KLH). The mAb DOB.L1 (anti-DO; mouse IgG2b isotype) has been generated by immunizing BALB/c mice with the C-terminal peptide from the HLA-DO β chain (RAQKGYYVTRQMSGNEVSRAVLLPQSC) coupled to KLH. Purified monoclonal antibodies were coupled to Sepharose as described (Vogt *et al.*, 1996).

Peptides

Peptides were synthesized and labeled at the N-terminus with the fluorophor 7-amino-4-methylcoumarin-3-acetic acid (AMCA; Lambda) as described (Kropshofer *et al.*, 1991). The following peptides were used: HA(307–319), PKYVKQNTLKLAT, from influenza hemagglutinin; HSP65(3–13), KTIAVDDEARR, from the 65 kDa heat shock protein of *Mycobacterium tuberculosis*; and E α (52–68), ASFEAAGALANIAVDKA, from the α -chain of murine I-E class II molecules.

Construction of a melanoma cell line expressing HLA-DO

The cDNAs encoding full-length HLA-DO α and β chains were generated by reverse transcription of RNA isolated from the Burkitt lymphoma cell line Raji, expressing HLA-DO (Tonnel *et al.*, 1985). After PCR amplification, the DO α and DO β cDNAs were cloned downstream of an SR α promoter (Takebe *et al.*, 1988) in expression vectors carrying resistance genes for hygromycin B (NTH2) and neomycin (NTNeo), respectively. The cDNAs were sequenced and corresponded to those previously described (Tonelle *et al.*, 1985; Trowsdale and Kelly, 1985).

M10 melanoma cells (Mackensen *et al.*, 1993) were transfected by electroporation (975 mF, 210 V) with a mixture of linearized NTH2-DO α and NTNeo-DO β plasmid DNA (30 μ g each). Cells resistant to both G418 (Gibco-BRL; 0.5 mg/ml) and hygromycin B (Boehringer Mannheim; 0.1 mg/ml) were selected. Expression of HLA-DO in this M10.DO cell line was assessed by RT-PCR.

Recombinant baculoviruses encoding HLA-DO α and β

The cDNA coding for HLA-DO β was modified by the C-terminal addition of an eight amino acid sequence (FLAG[®]) to allow purification of recombinant HLA-DO (rDO) by affinity chromatography on an anti-FLAG antibody column (Eastman Kodak). The cDNAs encoding full-length DO α and DO β were subcloned into the baculovirus transfer vector pVL1393 (Pharmingen). Recombinant baculoviruses were generated by homologous recombination following co-transfection of SF9 cells with recombinant transfer plasmids and linearized baculovirus BaculoGold DNA (Pharmingen). Preparation of virus stocks, culture and infections were performed as described (O'Reilly *et al.*, 1994).

Flow-cytometric analysis

Cells were stained with the anti-CLIP mAb Cer.CLIP or anti-DR mAb ML11C11 followed by goat anti-mouse IgG1-R-phycoerythrin (Southern Biotechnology Associates) and analyzed using a FACScan flow-cytometer (Becton-Dickinson).

Subcellular fractionation

Subcellular fractionation and marker analysis were performed as described (Kropshofer *et al.*, 1997b). Lysosomal fractions were shown to contain the lysosomal markers lamp-1 and β -hexosaminidase as well as HLA-DR, -DM and -DO molecules, but were devoid of invariant chain, class I molecules and the cation-independent mannose-6-phosphate receptor.

Purification of class II molecules

HLA-DR molecules were isolated from the respective T2 transfectants by affinity chromatography using anti-DR mAb L243, as described (Kropshofer *et al.*, 1995a). HLA-DM and HLA-DM-DO complexes were purified from human spleen solubilized in 1 or 2% NP-40 using the anti-DM mAb DM.K8, as described (Vogt *et al.*, 1996). HLA-DM devoid of DO was purified from T2.DR4.DM transfectants.

Purification of recombinant HLA-DO

rDO was purified from virus-infected SF9 cells at 72–96 h post-infection, essentially as described for HLA-DR (Kropshofer *et al.*, 1995a). Briefly, cell pellets were lysed for 1 h on ice in 20 mM Tris pH 7.4, 5 mM MgCl₂, 1% NP-40, including protease inhibitors. Supernatants cleared by ultracentrifugation were loaded on an anti-FLAG immunoaffinity column. After repeated washing, rDO was eluted with 100 mM sodium phosphate buffer, pH 11.0, 0.1% Zwittergent 3–12 (Calbiochem), neutralized and concentrated by ultrafiltration using a 20 kDa cut-off cellulose triacetate membrane (Sartorius).

Peptide-binding assay

Solubilized HLA-DR molecules and the respective AMCA-labeled peptide were co-incubated with or without detergent-solubilized HLA-DM and/or HLA-DO for various periods of time at 37°C in 50 mM sodium phosphate, 50 mM sodium chloride, 50 mM sodium acetate, 0.1% Zwittergent 3–12 (Calbiochem) pH 5.0. Binding was quantified by high performance size exclusion chromatography (HPSEC), as described previously (Kropshofer *et al.*, 1995a).

Mass spectrometry

DR-associated peptides were analyzed by MALDI-MS as described (Kropshofer *et al.*, 1995b). Affinity-purified DR molecules (5–10 μ g) were washed with double-distilled water in an Ultrafree ultrafiltration tube with a 30 kDa cut-off (Millipore). Peptides were eluted by incubation in 0.1% trifluoroacetic acid (TFA) for 0.5 h at 37°C. After separation of protein by ultrafiltration, peptides were lyophilized and re-dissolved in 0.1% TFA, 5 M 1,4-dihydroxybenzoic acid/acetonitrile (1:1). Spectra were recorded on a Finnigan LaserMat vision 2000 mass spectrometer and collected by averaging the ion signals from 40–60 laser shots.

Western blot analysis

Samples were separated by SDS-PAGE (12%) and transferred onto Immobilon PVDF membranes (Millipore). Binding of biotinylated antibody was detected by incubation with horseradish peroxidase-conjugated streptavidin (Dianova) followed by enhanced chemoluminescence with Super-Signal[™] or Super-Signal[™] Ultra (Pierce).

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